THE UNIVERSITY OF ALBERTA

STUDIES ON CULTURES OF RUMEN CELLULOLYTIC BACTERIA TREATED WITH ISONICOTINIC ACID HYDRAZIDE, A VITAMIN B-6 INHIBITOR

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on Cultures of Rumen Cellulolytic Bacteria Treated with Isonicotinic Acid Hydrazide, a Vitamin B-6 Inhibitor" submitted by James Ingham Elliot, B.S.A., in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A fermentation medium was developed in which ammonium sulphate was the sole source of nitrogen and a 4 per cent (weight per volume) suspension of purified wood cellulose, which had been ball-milled for 1 hour and heated in an autoclave at 15 pounds per square inch for 1 hour, was the sole source of energy and a major source of carbon. In experiments designed to obtain indirect evidence to indicate the occurrence of transamination in rumen cellulolytic bacteria, such bacteria were incubated in this medium in the absence and presence of isonicotinic acid hydrazide, a vitamin B_6 inhibitor. Growth, as measured by bacterial protein nitrogen increase, was satisfactory in the absence of the inhibitor, but essentially no growth occurred in cultures treated with isonicotinic acid hydrazide at a level of 0.1 millimolar, even when vitamin B_6 in the form of pyridoxine, pyridoxal, or pyridoxamine was added at levels of 1.0 or 1.5 millimolar either 21 hours before, at the same time as, or 6 hours after the addition of the inhibitor. Inasmuch as none of the three vitamin B_6 forms, at the levels employed, reversed the inhibition caused by isonicotinic acid hydrazide, it was concluded that this approach offered little promise as a means of obtaining even indirect evidence for the occurrence of transamination.

Subsequent experiments were devoted to study of cellulose utilization in cultures treated with isonicotinic acid hydrazide as measured by production of total volatile fatty acid and of carbon dioxide. The results indicated that, as compared to untreated positive controls or negative controls killed with a bactericide, treated cultures had an appreciable capacity to utilize cellulose. Thus, it appeared that lack of growth in treated cultures was not primarily attributable to inhibition of cellulose utilization in the presence of isonicotinic acid hydrazide.

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INTRODUCTION

Pure culture studies have shown that many ruminal cellulolytic bacteria can utilize ammonia as their sole source of nitrogen while others can derive a large part of their nitrogen requirement from ammonia. Vitamin B_6, which is known to be synthesized in the rumen, catalyzes a variety of metabolic reactions including transamination, a reaction which is of major importance in the synthesis of amino acids. This reaction is probably involved in the synthesis of amino acids by rumen microorganisms that utilize ammonia nitrogen. However, transamination has not been demonstrated in the rumen cellulolytic bacteria.

Inasmuch as vitamin B_6 is the cofactor required for the transamination reaction, it was thought that indirect evidence regarding the occurrence of transamination in rumen cellulolytic bacteria might be obtained by studying cultures of such organisms grown in a medium containing a vitamin B_6 inhibitor, and with ammonia as the sole source of nitrogen and cellulose as the major source of carbon.

LITERATURE REVIEW

Nitrogen Requirements of the Rumen Cellulolytic Bacteria

It is well established that organic and inorganic forms of nitrogen entering the rumen are degraded by the rumen microorganisms to ammonia and other products. Investigations on this subject are summarized in a review article by McLaren (1964) and in monographs by Annison and Lewis (1959) and Barnett and Reid (1961). Since large amounts of ammonia are formed in the rumen, it would be expected that the ruminal organisms could utilize this form of nitrogen for growth.

Gill and King (1958) reported that a strongly cellulolytic species of Butyrivibrio assimilated 70% of the free ammonia in the medium in the presence of amino acids. When the amino acids were deleted from the medium, no growth occurred. Histidine, isoleucine, methionine, lysine, cysteine, leucine, tyrosine, and valine were the nutritionally critical amino acids, and it was concluded that the organism had limited ability to synthesize these amino acids.

Reporting on the nutrition of five strains of Bacteroides succinogenes, Bryant et al. (1959) noted that ammonia as $(NH_4)_2SO_4$ was essential for their growth even in the presence of 18 L-amino acids, purines, pyrimidines, and B-vitamins. Growth was depressed in the presence of $(NH_4)_2SO_4$ alone, but the inclusion of cysteine in the medium overcame this depression. It was concluded that the function of cysteine was to supply sulphur.

Bryant and Robinson (1961) reported on the nitrogen requirements of several strains of cellulolytic bacteria that predominate in the rumen. Five strains of Ruminococcus albus and three strains of Ruminococcus flavefaciens demonstrated an absolute requirement for

ammonia nitrogen (supplied in this work as $(NH_4)_2SO_4$), even in the presence of 17 amino acids, purines, and pyrimidines. Bacteroides succinogenes S85, Ruminococcus albus strain 7, and Ruminococcus flavefaciens C-94 incorporated ammonia nitrogen in amounts equal to or greater than the amount of cellular nitrogen formed when grown in a medium containing both ammonia and 1% trypticase as sources of nitrogen. In contrast, a non-cellulolytic rumen organism, Streptococcus bovis, incorporated little ammonia nitrogen or produced ammonia when grown in the same medium.

Bryant and Robinson (1962a) reported on the assimilation of ammonia and C^{14} from labeled casein hydrolysate by a wide variety of cellulolytic and non-cellulolytic rumen organisms. Their results indicated that many species of predominant cellulolytic rumen bacteria prefer to synthesize their cellular constituents from ammonia nitrogen and carbon sources other than amino acids. Bryant and Robinson (1962b), studying the nutritional requirements of rumen microorganisms, found that 82% of freshly isolated strains grew well in media which contained ammonia and a small amount of cysteine as the sole sources of nitrogen.

In summary, it is apparent that many strains of ruminal cellulolytic bacteria have either an absolute or partial requirement for ammonia as a source of nitrogen for growth.

Synthesis of Amino Acids and Protein by Rumen Bacteria

Rumen microbial populations possess the ability to synthesize amino acids and protein given ammonia as their source of nitrogen. A great deal of indirect evidence to support this statement has accumulated as a result of feeding trials designed for other purposes. These are discussed by Annison and Lewis (1959) and Chalmers (1961). It is the

intent here to present only direct evidence from *in vivo* and *in vitro* studies which support this statement.

Wegner et al. (1940b)reported that during *in vitro* fermentations in which either ammonium bicarbonate or urea served as the sole source of nitrogen, the concentration of free ammonia nitrogen in the medium decreased. Since there was no change in the total nitrogen content of the system during the experimental period, they concluded that the observed decrease was not due to loss of ammonia from the medium. In a subsequent experiment, the fermentation mixture was separated by filtration into supernatant and residual filtrate fractions at the beginning and end of the experimental period. The residual filtrate nitrogen was assumed to represent bacterial protein. Using this technique these workers demonstrated that 90.6% of the free ammonia nitrogen lost during the experimental period was recovered as residual filtrate nitrogen. This was taken as proof of protein synthesis by the bacteria.

Quantitative conversion of urea nitrogen to ammonia nitrogen by rumen microorganisms *in vitro* was demonstrated by Pearson and Smith (1943a). Subsequently these workers (1943b) reported that, in 3-hr experiments, the decrease in the non-protein nitrogen content of their *in vitro* fermentation mixture was accompanied by an equivalent increase in trichloroacetic acid precipitatable nitrogen (TCA-N), which was assumed to represent bacterial protein nitrogen. There was no change in the total nitrogen content of the system throughout the experimental period.

Three sheep and two goats were fed a synthetic diet containing urea as the main source of nitrogen by Loosli et al. (1949). The animals were maintained for 20 days on the synthetic diet which contained low

levels of all the amino acids due to protein contamination of some of the ingredients. At the end of the 20-day adjustment period, the rumen contents were sampled. The samples were hydrolyzed and the hydrolysates assayed microbiologically for the ten amino acids essential for the growth of the rat. The rumen contents contained the ten essential amino acids in amounts nine to twenty times greater than those present in the synthetic diet. The authors concluded that the amino acids were synthesized by the rumen microorganisms from the ammonia nitrogen derived from urea.

Agrawala, Duncan, and Huffman (1953) and Duncan et al. (1953) fed fistulated calves a purified ration in which urea was the sole source of nitrogen. The experiments were designed to obtain quantitative data on the synthesis of protein and of ten amino acids by rumen microorganisms in vivo. In the first experiment, consisting of seven trials, Agrawala et al. (1953) reported increases of 33 to 109 g of true protein during the first 6 hr after feeding. During this period approximately 90% of the dietary urea had disappeared from the rumen. In the second experiment, using a microbiological assay, Duncan et al. (1953) assayed samples of rumen contents taken from calves before and 6 hr after feeding, for the ten amino acids essential for growth of the rat. The results, expressed as grams of amino acid in the total rumen contents, consistently demonstrated an increase in each of the amino acids.

Phillipson et al. (1962) incubated rumen liquor in vitro with NH_4Cl and noted that whenever there was a large decrease in the free ammonia nitrogen content of the fermentation mixture, there was a corresponding increase in the TCA-N content. Subsequently, an experiment employing $N^{15}H_4Cl$ demonstrated that a decrease in free ammonia nitrogen content of the fermentation mixture was accompanied by an increase in

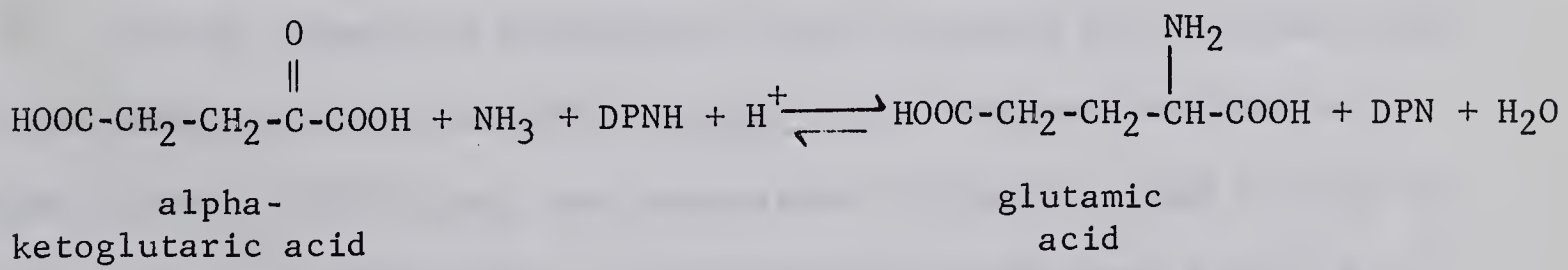
the N^{15} concentration in the TCA-N fraction. In a second experiment of this type, an increase in the free ammonia nitrogen of the fermentation mixture accompanied by a small increase in N^{15} concentration of the TCA-N was observed. The authors state: "There is no doubt that during incubation, decreases in the concentration of ammonia nitrogen indicate a synthesis of protein or simpler nitrogenous compounds that are precipitated by trichloroacetic acid; there seems no way in which it can occur except by the assimilation of ammonia-N with subsequent synthesis of amino acids and protein within the bacterial cells."

Routes of Incorporation and Utilization of Ammonia Nitrogen and the Associated Function of Vitamin B_6

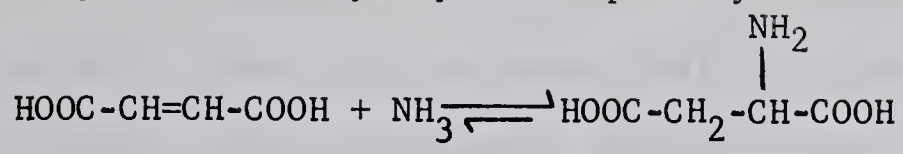
Despite convincing evidence to support the conclusion that amino acids and proteins are synthesized from ammonia nitrogen by rumen microorganisms, the mechanisms of incorporation and utilization of ammonia nitrogen are poorly understood. Most of the research reported on the subject of amino acid metabolism by rumen microorganisms is concerned with amino acid catabolism. McLaren (1964) has reviewed these reports.

There are several pathways by which ammonia nitrogen may be incorporated into cellular material, but only two of these involve incorporation of ammonia directly into the alpha-amino group characteristic of all primary amino acids. These pathways are given in equations 1 and 2.

1. Synthesis of glutamic acid by the glutamic acid dehydrogenase pathway



2. Synthesis of aspartic acid by the aspartase pathway



fumaric acid aspartic acid

Oginsky and Umbreit (1954) note both these pathways as occurring in bacteria and state that the synthesis of glutamic acid is the most important pathway. Both of the reactions are reversible and can occur under anaerobic conditions such as those present in the rumen. However, glutamic acid dehydrogenase activity has never been demonstrated in rumen microorganisms, and the aspartase system has never been demonstrated in the anabolic direction.

The reverse reactions result in the deamination of glutamic and aspartic acids, and the deamination of these amino acids has been demonstrated using rumen microorganisms in vitro (Lewis and Emery, 1962 and Hoshino and Hirose, 1963). The mechanisms involved were not studied by Lewis and Emery. Hoshino and Hirose obtained evidence to suggest that the enzyme responsible for the degradation of aspartic acid is an aspartase. These experiments were done with incubation mixtures consisting of rumen bacterial cells, a buffer, and a substrate. It is probable that in the presence of excess ammonia the synthesis of glutamic and aspartic acids would be favored. It is known that the equilibrium of the glutamic dehydrogenase system favors the formation of glutamic acid (White, Handler, and Smith, 1964).

Strong suggestive evidence for the occurrence of these reactions in the anabolic direction under in vivo conditions was obtained by Boggs (1959). $(N^{15}H_4)_2SO_4$ was administered through a rumen fistula to two sheep on a purified diet. Fractionation of the rumen fluid, 3 hr later, revealed that 67% of the N^{15} recovered was in the bacterial

fraction. Furthermore, analysis of the amino acids isolated after hydrolysis of the whole rumen fluid revealed that the atom per cent excess N^{15} in the amino acids was greatest in glutamic and aspartic acids. Excess N^{15} was also found in all the other amino acids isolated. The results were interpreted as supporting the theory of initial incorporation of ammonia nitrogen into glutamic and aspartic acids.

Once ammonia nitrogen has been incorporated, the other amino acids necessary for the synthesis of protein are formed directly or indirectly from the products of ammonia incorporation by a wide variety of synthetic pathways.

Transamination, defined in a review paper by Guirard and Snell (1964) as a reaction involving the transfer of an amino group from an amino acid to a keto acid without the intermediate formation of ammonia, is a major reaction involved in the synthesis of practically all the naturally occurring amino acids. Transaminases are found in all plant, animal, and bacterial cells (White, Handler, and Smith, 1964). These have never been demonstrated in rumen cellulolytic bacteria; however, McLaren (1964) and Barnett and Reid (1961) have indicated that the involvement of transaminases in the synthesis of amino acids by rumen microorganisms is highly probable.

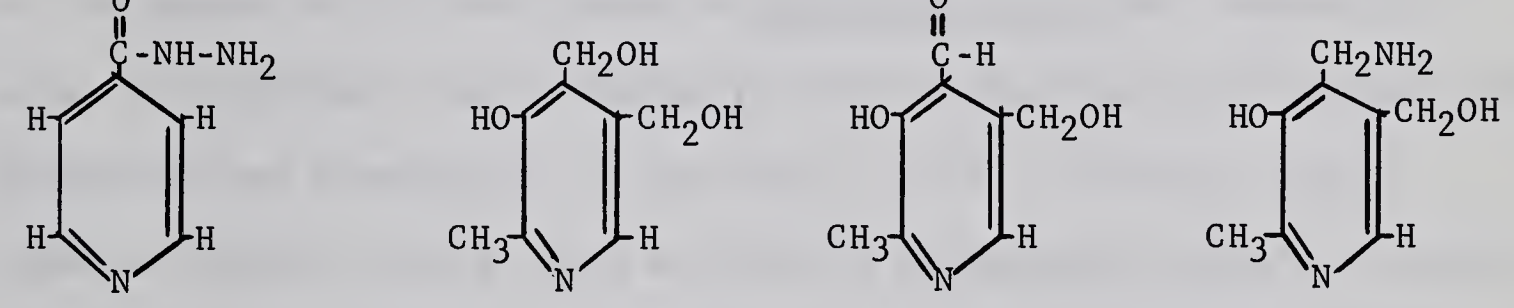
Vitamin B_6, a member of the B-complex known to be synthesized in the rumen, serves in its coenzyme form, pyridoxal-5-phosphate, as the cofactor for enzymatic transamination and for other reactions associated with both the anabolic and catabolic aspects of amino acid metabolism. In a review paper, Snell (1953) listed the general enzymatic reactions involved in the metabolism of amino acids for which vitamin B_6 serves as a cofactor as: (a) amino acid transaminases, (b) amino acid decarboxylases, (c) amino acid dehydrases and desulfhydrases, (d) amino acid

racemases, and (e) miscellaneous reactions involving amino acids and catalyzed by vitamin B_6.

Meister (1955), Snell (1958), Braunstein (1960), Guirard and Snell (1964), and King (1964) have published reviews on the subject of enzymatic transaminations, the involvement of vitamin B_6 in these reactions, and the importance of such reactions in the synthesis of amino acids at all phylogenic levels.

Isonicotinic Acid Hydrazide as an Inhibitor of Vitamin B_6 Dependent Reactions

The structural similarity (Fig. 1) between the members of the vitamin B_6 group (pyridoxine, pyridoxal, and pyridoxamine) and isonicotinic acid hydrazide (INH) suggests that this compound could act as a vitamin B_6 inhibitor.



isonicotinic acid hydrazide (INH) | pyridoxine | pyridoxal | pyridoxamine

Fig. 1. Structural similarity between INH and three vitamin B_6 forms

Structural similarities also exist between INH and niacin, but INH does not appear to affect the action of enzymes dependent on niacin as a cofactor. Boone and Woodward (1953) screened a series of B-vitamins, including pyridoxine and niacin, to determine their ability to reverse the effects of INH on Escherichia coli, Lactobacillus plantarum, Streptococcus pyogenes, Saccharomyces carlsbergensis, Mycobacterium tuberculosis

H37Rv, and Mycobacterium butyricum. The growth of all the organisms except Streptococcus pyogenes was inhibited by INH. Reversal of the growth inhibition was achieved in all the organisms except the Mycobacteria, but only members of the vitamin B_6 group were effective in reversing the inhibition. In a similar experiment, Pope (1956) tested the ability of a variety of B-vitamins, amino acids, purine and pyrimidine bases, keto acids, and other compounds to reverse the growth inhibiting effect of INH on Mycobacterium tuberculosis H37Rv. Only pyridoxal and pyridoxamine, of all the B-vitamins tested, were effective in antagonizing the effects of INH; niacin had no effect.

Tryptophanase, an enzyme catalyzing the degradation of tryptophan to indole, pyruvic acid, and NH_3, requires the coenzyme pyridoxal-5-phosphate for its activity. The functioning of this enzyme, as measured by the production of indole when an Escherichia coli cell suspension acted on tryptophan, was inhibited by INH (Yoneda, Kato, and Okajima, 1952). Pyridoxine was antagonistic to the effect of INH. Lichstein (1955) reported similar results using a strain of Escherichia coli that required vitamin B_6 for growth; he observed that the degree of protection afforded by the three vitamin B_6 forms against the inhibitory effects of INH was dependent on the concentrations of INH and vitamin B_6 used.

Yoneda and Asano (1953) studied the effect of INH on the arginine decarboxylase of Escherichia coli. This enzyme is known to require pyridoxal-5-phosphate as its coenzyme. Exposure of a washed-cell suspension of the organism to 0.01 M INH for 30 min prior to the addition of the substrate, significantly reduced the ability of the organism to decarboxylate arginine. Addition of pyridoxal at the end of the period of exposure to INH restored 63% of the activity.

Hicks (1961) studied the effect of INH on the growth and transaminase activity of Escherichia coli strain 15, mutant M2, and found that although INH inhibited the growth of this organism it had no effect on the transaminase activity of whole cell suspensions. However, when cell-free extracts were prepared, INH inhibited their transaminase activity.

Investigating the action of INH on the ability of several amino acids to transaminate with alpha-ketoglutaric acid in cell-free extracts of resistant and susceptible strains of Mycobacterium tuberculosis, Youatt (1958) reported that all the reactions studied were inhibited by INH. Pyridoxal-5-phosphate in equimolar concentration with INH prevented inhibition. Similarly, Hicks and Cymerman-Craig (1957) reported the inhibition by INH of an alanine-alpha-ketoglutaric transaminase isolated from pig-heart muscle.

In summary, INH is an effective inhibitor of a variety of vitamin B_6 dependent reactions involved in amino acid metabolism in a variety of organisms.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

Preliminary studies. Development of an *in vitro* fermentation procedure and medium.

Experiment 1. Determination of the minimum effective concentration of isonicotinic acid hydrazide.

Experiment 2. Determination of the effect of three vitamin B_6 forms on cultures inhibited with isonicotinic acid hydrazide.

Experiment 3. Determination of the volatile fatty acid and CO_2 production of cultures treated with isonicotinic acid hydrazide.

Preliminary Studies

Preliminary studies were carried out in two parts. In the first part, consisting of runs 1 to 11, attempts were made to apply the medium and fermentation procedure of Winter (1962) unmodified; the results obtained failed to approximate those of Winter (Table 1). In the second part, consisting of runs 12 to 16, attempts were made to obtain improved growth by modifying the medium and fermentation procedure of Winter and to obtain more complete precipitation of bacterial protein nitrogen (BPN) by using $Zn(OH)_2$ rather than trichloroacetic acid as the precipitating agent.

Table 1

Mean results obtained in runs 1 to 11 vs. those reported by Winter

	Runs 1 - 11	Winter (TCA-N)
BPN present at 24 hr (mg/100 ml)	13.9[1]	25.7
Urea hydrolysis at 24 hr (runs 6 - 11) %	85[2]	98
Range of urea hydrolysis (runs 6 - 11) %	39 - 100	97 - 98

[1]Average of 44 fermentations.

[2]Average of 24 fermentations.

Development of Methods, Runs 12 to 16

From the results obtained in runs 1 to 11 it was apparent that the growth obtainable by application of the methods described by Winter (1962) was so low as to preclude any possibility of detecting differences attributable to the treatments planned. A number of modifications of the

methods of Winter were therefore studied in an attempt to develop satisfactory methods and procedures.

It was recognized that changes in methods and procedures might result in the growth of different types of organisms. As a means of detecting any such changes that might occur, smears were made of the inocula and of the flask contents at appropriate times during fermentation throughout this phase of the investigation. These were stained by the Gram method. Examination of the smears* indicated that the same gram-negative bacteria appeared to proliferate in all cases, but it was not clear whether they were micrococci or very small rods. The bacteria which proliferated were present in the original inocula. Other investigators, Dehority, El-Shazly, and Johnson (1960), El-Shazly et al. (1961), and Dehority (1963) have reported the proliferation of gram-negative or gram-variable cellulolytic micrococci under conditions generally comparable to those of the present study.

Reasons for studying the various modifications tested and the results obtained on testing are presented in sequence in the next few pages.

Run 12

Run 12 was designed to obtain information on: (a) the pH of the fermentation medium at several times prior to inoculation as well as the effect of adjusting the pH of the medium to 6.8 to 6.9 prior to inoculation on the amount of BPN measurable at 24 hr, and (b) the effect of substituting $Zn(OH)_2$ for trichloroacetic acid as the protein precipitant on the amount of BPN measurable in duplicate samples of six 24-hr

*The smears were examined by Dr. J. N. Campbell of the Department of Microbiology, University of Alberta. His generous cooperation is most gratefully acknowledged.

fermentations.

Hydrogen ion concentration

Regarding the preparation and treatment of his flasks, Winter (1962) states: "The fermentation flasks were placed in a water bath.... Prior to inoculation the fermentation flasks were gassed with carbon dioxide for at least fifteen minutes. After inoculation the fermentation was allowed to proceed for twenty-four hours and during that time the pH was determined three or four times...." This suggested that the first pH determination was made some hours after inoculation and therefore in the first 10 runs of the present study the first determination was made approximately 6 hr after inoculation at which time the pH was usually in the range of 6.5 to 6.9 and considered normal. In run 11 the pH of the flasks happened to be determined just before inoculation and was found to be in excess of 7.0 in most cases. The occurrence of a pH much greater than 7.0 at the time of, or for any length of time after inoculation, could be detrimental to the growth of organisms in the inoculum. The effect of adjusting the pH of the flasks to 6.8 to 6.9 before inoculation on the amount of BPN measurable at 24 hr was therefore studied.

The pH of the medium during preparation was consistently in excess of 7.0 (Table 2) to an extent which could have been detrimental to the growth of organisms inoculated into a medium of this pH. Adjusting the pH of the medium to 6.8 to 6.9 with 2N HCl before inoculation resulted in a desirable pH immediately after inoculation (Table 2).

Table 2

Hydrogen ion concentration of the medium during preparation and immediately after inoculation

pH determined in	No. of determinations	Mean	Range
Basal medium		9.4	
Basal medium plus cellulose suspension	12	9.7	
Complete medium after passage of CO_2 for 15 - 30 min	12	8.8	7.2 - 9.9
Fermentation mixture after inoculation	12	6.8	6.7 - 7.1

Adjustment of the pH before inoculation did not increase the amount of BPN measurable at 24 hr. At the end of the fermentation period an average of 13.9 mg BPN/100 ml were present. This was not different from the average amount present at this time in runs 1 to 11 (Table 1, p. 13). Thus, failure of the medium and fermentation procedure of Winter (1962) to give satisfactory results in the present study could not be attributed to the pH of the fermentation flasks at the time of inoculation. However, since it was planned to investigate the replacement of urea with $(NH_4)_2SO_4$ in a future trial, the maintenance of a pH of less than 7.0 in the fermentation medium was considered necessary to prevent losses of ammonia from the medium and to overcome the possible toxic effects of free ammonia. The procedure for preparing the fermentation flasks with respect to pH control was altered accordingly and is outlined in the General Experimental section to follow.

Zinc hydroxide vs. trichloroacetic acid

In runs 1 to 11 in which bacterial protein was precipitated with

trichloroacetic acid according to the method of Cline, Hershberger, and Bentley (1958), a cloudy supernatant indicative of incomplete precipitation often remained after centrifugation. A similar observation was reported by Winter (1962) who found that when trichloroacetic acid was replaced by tungstic acid as the protein precipitant, 16.6 mg/100 ml more BPN was recovered. Early in the present study it was noted that the deep yellow acid insoluble tungstate made it very difficult to visibly determine the completion of the digestion stage of the micro-Kjeldahl nitrogen analysis. Therefore the possibility of replacing trichloroacetic acid with another protein precipitant, $Zn(OH)_2$, was studied.

With one exception, $Zn(OH)_2$ precipitated more BPN than did trichloroacetic acid (Table 3), but the difference between the means was not significant. The cloudy supernatants observed in runs 1 to 11 and in run 12 after centrifugation of samples precipitated with trichloroacetic acid were not observed when $Zn(OH)_2$ was the protein precipitant. This indicated that the use of $Zn(OH)_2$ resulted in a more complete precipitation of bacterial protein. Furthermore, $Zn(OH)_2$ is soluble under the acid conditions of the micro-Kjeldahl digestion procedure and does not interfere with the visible determination of the completion of digestion. For these reasons $Zn(OH)_2$ was used as the protein precipitant in all subsequent work.

Table 3

Trichloroacetic acid vs. zinc hydroxide as bacterial protein nitrogen precipitants

Flask	Duplicate 1 trichloroacetic acid	Duplicate 2 $Zn(OH)_2$
	mg BPN/100 ml	
1	13.8	14.2
2	9.6	8.8
3	14.2	16.9
4	14.5	16.4
5	16.6	20.3
6	14.8	18.0
mean	13.9	15.8

Runs 13 and 14

In run 13, only 30% of the urea, as determined by the method of Brown (1959), in the medium was hydrolyzed during the fermentation. Essentially 100% of the urea was hydrolyzed in runs 6, 7, 8, and 11 but only 39% and 72% was hydrolyzed in runs 9 and 10 respectively. The fact that the average amount of BPN present at 24 hr was 12.0 mg/100 ml for runs 9 and 10 as compared to 15.3 mg/100 ml for runs 6, 7, 8, and 11 suggested the possibility that variations in the rate or extent of urea hydrolysis associated with possible differences in the ureolytic activity of different inocula might be an uncontrolled cause of variation in the results obtained with procedures employed to this point.

For optimum growth conditions, nitrogen in a form that can be utilized by the bacteria should be readily available at all times. Bryant and Robinson (1961) have reported that rumen cellulolytic bacteria that

utilized ammonia nitrogen took up ammonia nitrogen in amounts equal to, or greater than, the amount of cellular nitrogen synthesized. When urea is the source of nitrogen, the availability of nitrogen is dependent on the rate or extent of the hydrolysis of urea to NH_3 and CO_2. $(NH_4)_2SO_4$ supplies nitrogen in a form which is readily available; the possibility that it might be a more reliable source of nitrogen than urea was therefore tested in run 14. Isonitrogenous levels of urea and $(NH_4)_2SO_4$ were employed in runs 13 and 14 respectively; the results are summarized in Table 4.

Table 4

The effect of two nitrogen sources, urea and ammonium sulphate, on the amount of bacterial protein nitrogen measurable at 24 hours

Nitrogen source	Urea	$(NH_4)_2SO_4$
Run	13	14
	mg BPN/100 ml	
Flask 1	12.8	20.9
2	14.8	21.6
3	17.1	19.4
Mean	14.7	20.6

The amount of BPN recovered at 24 hr was 40% greater (significant at $P < 0.05$) in run 14 than in run 13. On the basis of these results, $(NH_4)_2SO_4$ was used as the source of nitrogen in all subsequent runs.

The modifications made up to this point increased the amount of BPN synthesized to 80% of that reported by Winter (1962) after a 24-hr fermentation period.

Runs 15 and 16

In runs 1 to 14 the cellulose substrate was prepared by ball-milling a 4% (w/v) suspension of Solka Floc BW-40 for 1 hr. Seventy-five ml of this suspension were added to each fermentation flask. The effects of heating the ball-milled cellulose suspension in an autoclave at 15 psi for 1 hr and of increasing the fermentation period to 36 hr were studied in runs 15 and 16. The results, with those of run 14 included for comparison, are summarized in Table 5.

Table 5

The effects of heating the cellulose suspension in an autoclave at 15 pounds per square inch for 1 hour and of increasing the fermentation time to 36 hours

Run		14	15	16	
Treatment		Nil	Autoclave	Nil	Autoclave
		mg BPN/100 ml*			
Time (hr)	0	8.6	9.2	13.7	13.7
	12	8.2	7.8	12.0	11.9
	18	10.1	18.8	12.8	15.8
	24	20.6	35.7	22.6	25.9
	30		46.6	30.1	35.9
	36		50.6	41.2	46.7

*Each value is the mean of three fermentations.

Significantly ($P < 0.05$) more BPN was present at 24 hr in run 15 than in run 14, but in run 16 the increase in BPN associated with heat treatment of the cellulose suspension was not significant. However, analysis of the pooled results for flasks, in which untreated cellulose

was used in runs 14 and 16 vs. those for flasks in runs 15 and 16 in which heat-treated cellulose was used, indicated that significantly ($P < 0.01$) more BPN was formed at 24 hr when heat-treated cellulose was used. Furthermore, the data in Table 5, p. 20, for BPN at 18 hr suggest that bacterial growth was initiated earlier in flasks containing heat-treated cellulose than in those in which untreated cellulose was used.

In a review article on the absorption of water by cellulose and starch, Urquart (1959) notes that under conditions of unlimited water availability, such as exist in a 4% suspension of cellulose and water, the hygroscopicity of cellulose and the cross-sectional area of the cellulose fibres increase with temperature. Increased cross-sectional area denotes increased surface area of the whole fibre and an increase in the size of the spaces between the component micelles of the cellulose fibre; the net result is an increased availability of the cellulose for microbial degradation. For these reasons, plus the results discussed above, treatment of the cellulose suspension in an autoclave at 15 psi for 1 hr was included in the procedure for all subsequent runs.

Increasing the fermentation period increased the amount of BPN recoverable at the end of the fermentation period (Table 5). This was to be expected; all subsequent runs were carried out using a 36-hr fermentation period.

Summary

1) In runs 1 to 11, attempts were made to apply the medium and fermentation procedure of Winter (1962). The resulting growth was not considered adequate to permit the detection of differences in growth that might be attributable to the treatments planned.

2) In runs 12 to 16, attempts were made to obtain improved growth and more complete recovery of BPN by modifying the medium and procedures used in runs 1 to 11. Each of the following modifications offered some advantage and was employed in the methods and procedures summarized under General Experimental, pp. 24 to 29.

a) adjustment of the pH of the fermentation medium prior to and after inoculation

b) replacement of trichloroacetic acid with $Zn(OH)_2$ as the bacterial protein precipitant

c) replacement of urea iso-nitrogenously with $(NH_4)_2SO_4$ as the source of nitrogen

d) heat treatment of the cellulose suspension in an autoclave at 15 psi for 1 hr

e) extension of the fermentation period from 24 to 36 hr

By listing and averaging the figures available for the amounts of BPN present at 0, 12, 18, 21, 24, 27, 30, or 36 hr from runs 1 to 11, 15 and 16, and 15 to 26, the curves presented in Fig. 2, p. 23, were derived. Curve A represents the average growth obtained with the medium and fermentation procedure of Winter (1962). Curve B, representing the average growth obtained in runs 15 and 16 using the heat-treated cellulose suspension, is included because these were the first runs in which all the modifications listed in the summary were employed, and certain decisions affecting Experiment 1 were based on these results. Curve C, included for comparative purposes, represents the average growth achieved in all runs in which the modified methods and procedures were used.

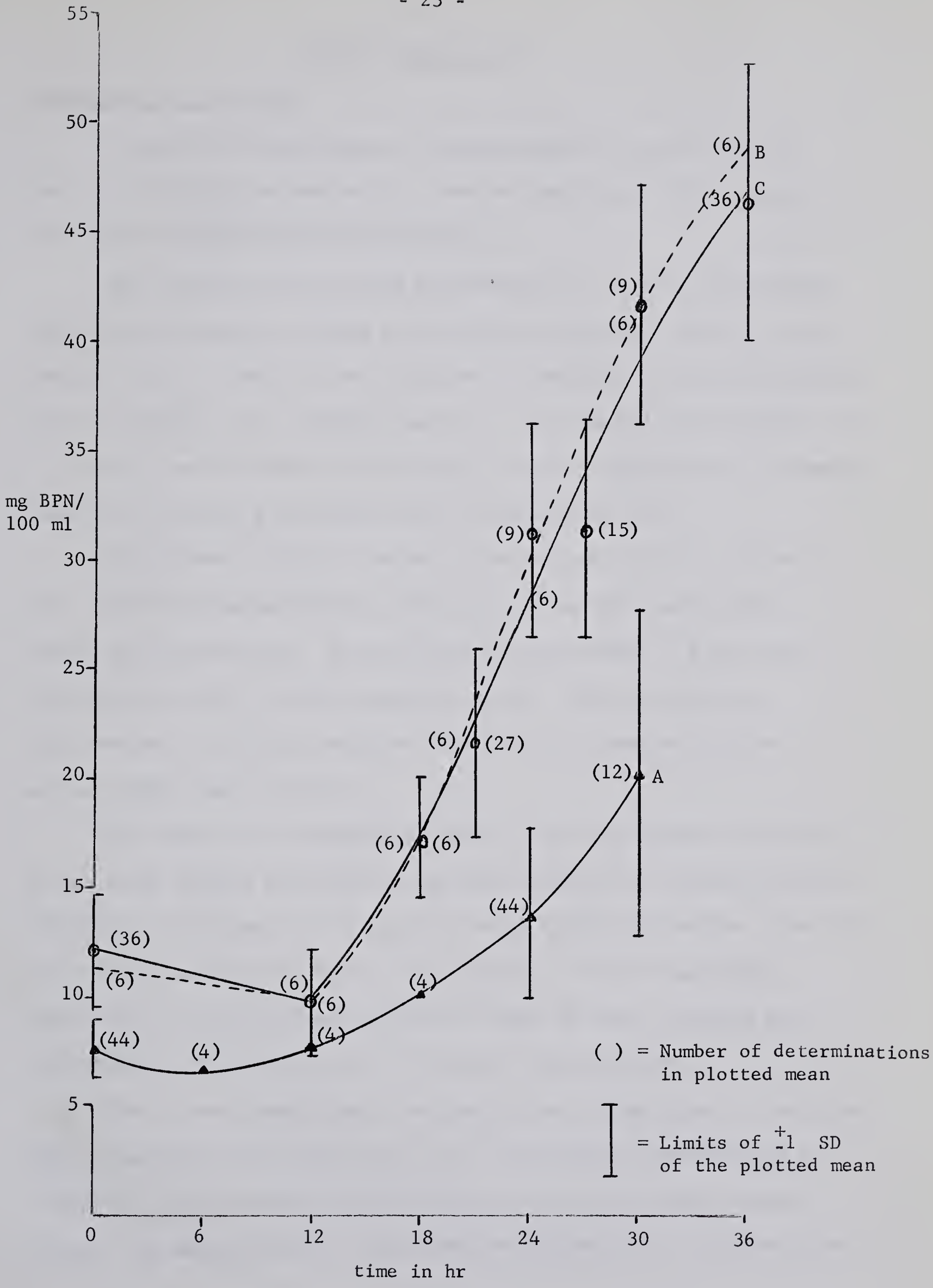
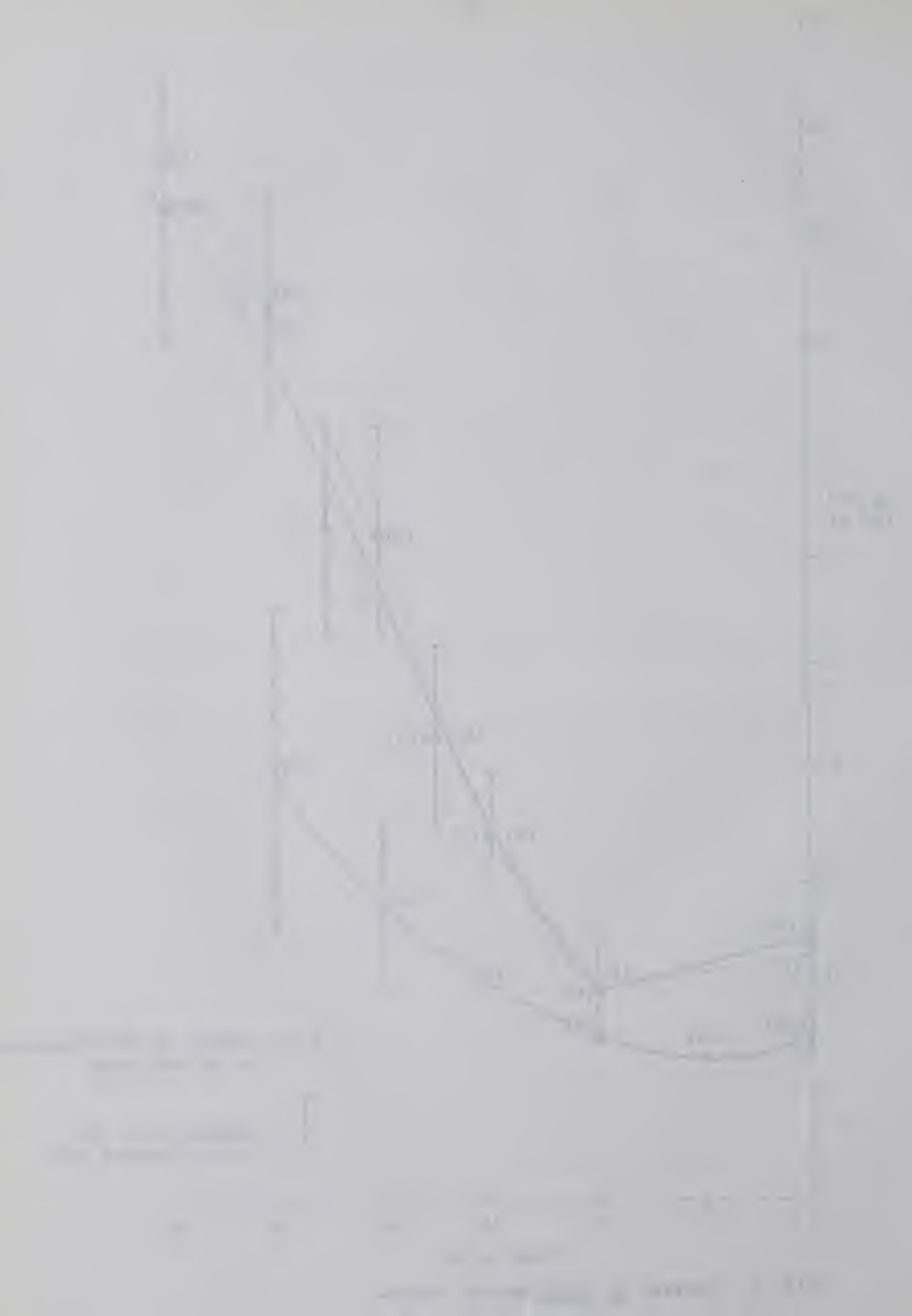


Fig. 2. Average <u>in vitro</u> growth curves.



General Experimental

Preparation of Inoculum

A rumen fistulated Jersey cow maintained on a ration of good quality alfalfa hay served as the source of inoculum; 15 lb. of hay were fed at 7:00 AM and at 6:00 PM daily.

The inoculum was collected approximately 4 hr after the morning feeding and prepared according to the method of Johnson, Dehority, and Bentley (1958). The buffer was prepared by heating a solution containing 1.059 g Na_3HPO_4, 0.436 g KH_2PO_4, and 0.1 g cysteine HCl per litre for 1 hr at 15 psi in an autoclave and allowing it to cool overnight in a stoppered flask in an incubator thermostatically controlled at 38 C.

Approximately 10 lb. of rumen contents were collected, placed in three layers of cheesecloth in a fruit press, and the rumen liquor expressed and discarded. The resulting pulp was mixed in a pail with approximately 1800 ml of the phosphate buffer. This mixture was repressed and the fluid, hereafter referred to as phosphate buffer extract (PBE), was collected.

The buffer, at a temperature of 38 C, was transported to the farm in two large thermos flasks which had been stored in an incubator thermostatically controlled at 38 C prior to being filled with buffer. The PBE was transported from the farm to the laboratory in the same flasks. Approximately 25 min elapsed between the time the rumen contents were collected and time of arrival of the PBE at the laboratory.

The PBE was centrifuged in a Sharples centrifuge (type T-1 equipped with a separator bowl fitted with a no. 5 ring dam for operation as a clarifier) at approximately 30,000 rpm until no fluid flowed from the outlet. The material from 1 inch above the bottom of the celluloid liner

was collected and resuspended in 300 ml of the phosphate buffer. This inoculum was dispensed to the in vitro fermentation flasks at the rate of 5 ml per flask with the aid of an automatic pipette[1].

Preparation of the Fermentation Medium

Table 6

Composition of the fermentation medium

Constituent	mg or ml/100 ml	g or ml/30 flasks
A. Solka Floc BW-40[a]	75.0 ml	
B. 1. $(NH_4)_2SO_4$	277.0 mg	8.31 g
2. $FeCl_3$ (4.4 mg/ml)		30.0 ml
$CaCl_2$ (5.29 mg/ml)	1.0 ml	
3. Na_2CO_3 (200 mg/ml)	1.0 ml	30.0 ml
4. Mineral mix[b]	4.0 ml	120.0 ml
5. Biotin (10 µg/ml)	2.0 ml	60.0 ml
6. PABA (100 µg/ml)	0.5 ml	15.0 ml
7. n-valeric acid (25 mg/ml)	1.0 ml	30.0 ml
8. H_2O (distilled)	5.5 ml	165.0 ml[c]
Final volume of B	15.0 ml	450.0 ml
Volume of A + B	90.0 ml	
Inoculum	5.0 ml	
pH adjusted, additives, wash	5.0 ml	
Final volume in each flask	100.0 ml	

[a] 4% w/v aqueous suspension.

[b] Na_2HPO_4, 56.5 g; NaH_2PO_4, 54.5 g; KCl and NaCl, each 21.5 g; $MgSO_4 \cdot 7H_2O$, 5.82 g; Na_2SO_4, 7.5 g -- all in 2000 ml of distilled water.

[c] Includes 15 ml as rinse with $(NH_4)_2SO_4$.

[1] Ivan Sorvall Inc., Norfolk, Conn.; model 4-10.

A. Preparation of the cellulose suspension

The purified wood cellulose used was Solka-Floc BW-40[1] prepared by a modification of the procedure described by Winter (1962). The 4% (w/v) suspension was ball-milled for 1 hr, transferred to a large flask, and heated in an autoclave at 15 psi for 1 hr.

B. Preparation of the other nutrients

The nutrient medium used was that described by Winter (1962) with $(NH_4)_2SO_4$ substituted iso-nitrogenously for urea. The composition of the basal nutrient medium is given in Table 6, part B. It was prepared as follows: constituents B-2, 3, 4, 5, 6, and 7 were mixed in amounts sufficient for 30 flasks. To this mixture 150 ml of distilled water were added and the pH was adjusted to 6.8 with concentrated HCl; $(NH_4)_2SO_4$ was added slowly from a weighing bottle and the bottle rinsed with 15 ml of distilled water to bring the total water added to 165 ml as per B-8. A magnetic stirrer was used to facilitate mixing. Adjustments in pH were made with the aid of a pH meter[2] and probe electrode assembly[3].

Preparation of the Fermentation Flasks

Fermentations were carried out in 125 ml Erlenmeyer flasks calibrated to 100 ml. Each flask was fitted with a two-holed rubber stopper through which passed gas inlet and outlet tubes. The gas inlet tubes were adjusted so that they extended almost to the bottom of the flasks to assure complete gassing of the fermentation mixture.

To each flask 75 ml of the 4% cellulose suspension were added by graduated cylinder and 15 ml of the basal nutrient medium were added by

[1]Lee Chemicals Ltd., 1119 Yonge St., Toronto 9, Ont.

[2,3]Beckman Instruments Inc., Edmonton, Alta.; model 76 expanded scale and cat. no. 39166, respectively.

pipette. Additives were introduced in a 1 ml volume either prior to or during the fermentation period according to the experimental plan.

The flasks containing the fermentation medium were placed in a water bath thermostatically controlled at 39 C, and CO_2 was passed through them at the rate of 60 to 120 bubbles per min. After 30 min each flask was removed individually to check the pH, and if necessary, readjust to 6.8 to 6.9 with saturated Na_2CO_3 or 2N HCl before replacing in the water bath. To maintain temperature and gaseous equilibrium, the flasks were left in the water bath while the inoculum was being prepared. They were inoculated with 5 ml of the inoculum previously described.

Fermentation Procedure

Twenty-four fermentation flasks representing three replicates of eight treatments were prepared. In addition, three flasks were prepared to serve as zero time controls.

Immediately after inoculation, the pH of each flask was again checked and, when necessary, readjusted to pH 6.8 to 6.9 with saturated Na_2CO_3. Fermentation was allowed to proceed for 36 hr during which time the pH was readjusted to pH 6.8 to 6.9 at 6-hr intervals.

Samples were taken at various times throughout each run as dictated by the particular experiment. Prior to taking the first sample from any one flask, the volume was adjusted to 100 ml with distilled water. When the experimental plan required the addition of a compound to the flasks after samples had been taken, a separate aqueous solution of the compound was prepared so that the required amount could be added to the flasks in a volume of 1 ml per flask. A 10-ml syringe fitted with an adapter and a short piece of polyethylene tubing was used for sampling. The flask contents were thoroughly mixed by pumping the syringe several times

before withdrawing the sample.

Bacterial Protein Nitrogen Analysis

For the purposes of this thesis, BPN is defined as the nitrogen precipitated by $Zn(OH)_2$ as determined by the micro-Kjeldahl procedure.

A. Precipitation procedure

Bacterial proteins were precipitated from 5-ml samples of the flask contents with $Zn(OH)_2$ using the reagents and method of Brown (1959). After standing overnight at room temperature, the precipitates were removed by centrifugation at 2300 rpm for 20 min. The supernatant solutions were decanted and stored at approximately 4 C in a cold room. The precipitates were washed once with 10 ml of distilled and demineralized water and recentrifuged at 2300 rpm for 10 min. The supernatant solutions were discarded and the precipitate quantitatively transferred to 100-ml micro-Kjeldahl flasks for nitrogen determination.

B. Micro-Kjeldahl nitrogen determination

Bacterial protein nitrogen was determined by the micro-Kjeldahl method with the following modifications of the AOAC (1960) procedure.

1) Digestion

The amount of H_2SO_4 used in the digestion mixture was increased to 5 ml to ensure that sufficient free acid was present throughout the digestion period.

2) Distillation

Distillation of the digested samples was carried out in a micro-Kjeldahl steam distillation head[1] fitted with a 500-ml three-necked, round-bottomed flask which served as a steam generator. Heating was

[1]Cave and Co. Ltd., Edmonton, Alta.; cat. no. 63840.

accomplished with electric heaters, rheostatically controlled. The distillate was trapped in 10 ml of 4% (w/v) H_3BO_3. Approximately 30 ml of distillate were collected and titrated with standard HCl.

C. Calculations

Results were expressed as mg BPN/100 ml and were calculated as follows: mg BPN/100 ml = ml HCl x N HCl x 14.008 x $\frac{100}{X}$;

X = volume in ml of sample analyzed.

D. Precision of the method

The precision of the method was determined by analysis of six samples from each of two flasks representing a normal 36-hr fermentation and a zero time control. The samples were prepared and analyzed as previously described; the coefficient of variation for each set of results was calculated. These were 0.5% and 8.7%, respectively. The high coefficient of variation for the zero time controls is attributed to unavoidable sampling errors caused by the large amount of undigested cellulose present at this stage in relation to the amount of BPN.

Statistical Analysis

The statistical treatment of the data was carried out following procedures described by Steel and Torrie (1960).

Experiment 1

Introduction

Exploratory tests were carried out in conjunction with runs 15 and 16 of the Preliminary Studies to obtain information on the effect of treating cultures with INH at a level of 3 mM at the time of inoculation and at different intervals after inoculation. The inhibitor was added to sets of three flasks at 0, 18, or 24 hr in run 15 and at 12, 18, or 24 hr in run 16.

Table 7

Estimated changes to 36 hours in bacterial protein nitrogen in flasks treated with isonicotinic acid hydrazide at 3 millimolar concentration at 0, 12, 18, or 24 hours compared with changes in control flasks during the same periods

		Controls	Treated			
No. of flasks		3*	3	3	3	3
INH added at		nil	0 hr	12 hr	18 hr	24 hr
		mg BPN/100 ml				
Run 15	0 hr	9.2	9.2			
	18 hr	18.8			18.8	
	24 hr	35.7				35.7
	36 hr	50.6	10.4		22.2	33.3
Estimated change in BPN content after treatment			+1.2		+3.4	-2.4
Estimated change in BPN content of controls from time of treatment			+41.4		+31.8	+14.9
Run 16	12 hr	11.9		11.9		
	18 hr	15.8			15.8	
	24 hr	25.9				25.9
	36 hr	46.7		11.3	15.9	26.9
Estimated change in BPN content after treatment				-0.6	+0.1	+1.0
Estimated change in BPN content of controls from time of treatment				+34.8	+30.9	+20.8

*Three control flasks for each time at which INH was added to three paired treated flasks.

Increases in the BPN content of treated flasks between the time of treatment and 36 hr were not significant. These increases, with the corresponding increases in the BPN content of the untreated control flasks included for comparison, are given in Table 7. Thus, at a concentration of 3 mM, INH was an effective growth inhibitor regardless of the time of addition.

In a subsequent experiment (Experiment 2 to follow) it was planned to study the relationship, if any, between INH and three vitamin B_6 forms. It was felt that the results of such an experiment would be most meaningful if the level of INH employed was minimal yet fully effective.

In consideration of the advantages of applying treatments to actively growing cultures, the growth curve for runs 15 and 16 (curve B, Fig. 2, p. 23) was examined. In these runs the logarithmic phase of growth appeared to be well established at 21 hr and this time was therefore selected as the most desirable one at which to treat the flasks. That the choice was a good one, is illustrated by the fact that in all subsequent runs the organisms were in the logarithmic phase of growth at 21 hr (curve C, Fig. 2, p. 23). Experiment 1, consisting of runs 17, 18, and 19, was designed to determine the minimal concentration of INH that would give essentially complete inhibition when added to cultures at 21 hr.

Experimental

Fermentation flasks were prepared and inoculated as described under General Experimental; the fermentation was allowed to proceed for 21 hr before addition of the inhibitor. Solutions of INH in distilled water were prepared in concentrations such that the addition of 1 ml per flask would give the desired concentration in the flask. The INH

solutions were added to the flasks, three flasks per treatment, and the flask contents thoroughly mixed. The fermentations were allowed to continue to 36 hr. Samples were taken from three untreated flasks at 21 hr and from an additional three untreated flasks at 36 hr to serve as 21 and 36-hr controls. At 36 hr, all flasks were sampled and the amount of BPN present determined. Results were expressed as per cent inhibition calculated by the following formula:

$$\text{Inhibition } \% = 100 \times \frac{(A-B) - (C-B)}{A-B}$$

A = average BPN (mg/100 ml) present in 36-hr controls
B = average BPN (mg/100 ml) present in 21-hr controls
C = average BPN (mg/100 ml) present in treated flasks at 36 hr

In calculating the per cent inhibition, the assumption was made that at 21 hr the amount of BPN present in all flasks within a given run was essentially the same. The coefficient of variability for the amount of BPN present at 21 hr within a given run was calculated and found to be 6.5%. This figure serves as a measure of the validity of the assumption made.

Results

At INH concentrations of 0.06 mM and greater, inhibition of growth was essentially complete (Table 8); the average inhibition calculated from the results for these concentrations was 99% with a coefficient of variation of 5%. At INH concentrations of 0.02 and 0.04 mM, slight growth occurred, while at concentrations of 0.01 mM and lower there was essentially no inhibitory effect.

Table 8

Per cent inhibition resulting from the presence of different concentrations of isonicotinic acid hydrazide in the fermentation flasks from 21 to 36 hours

Run	Concentration of INH	Inhibition
	mM	%
17	3.0	90
17	2.0	98
17	1.0	95
18	1.0	107
17	0.5	99
18	0.25	96
19	0.2	98
18	0.1	95
19	0.1	104
19	0.08	101
19	0.06	106
19	0.04	88
19	0.02	85
18	0.01	0
18	0.001	7
18	0.0001	0
18	0.00001	7

Discussion

On the basis of these results it appeared that INH at a final concentration of 0.05 mM would be the minimum effective level, however, when this concentration of INH was used in run 20, only 26% inhibition of growth occurred. In the same run, two control groups treated with INH at levels of 0.1 and 0.01 mM, respectively, were included. Growth was completely inhibited in the group containing the 0.1 mM level while no growth inhibition occurred in flasks containing the 0.01 mM level. In view of these observations and the results obtained with INH levels of 0.1, 0.06, 0.04, and 0.02 mM (Table 8), it was concluded that the growth inhibiting effect of INH was variable at levels between 0.1 and 0.02 mM.

The level of INH chosen as likely to be the minimum effective

level for use in all subsequent runs was 0.1 mM. This level had resulted in essentially complete inhibition in runs 18 and 19 (Table 8) and run 20. Isonicotinic acid hydrazide at this level was added at 21 hr in runs 18, 19, and 20 discussed above, and subsequently in runs 21, 22, 23, 25, and 26. The average per cent inhibition resulting from the use of this level in these runs was 99%, thereby justifying the choice of this level.

In run 24 INH was added to a group of flasks at 24 hr to give a concentration of 0.1 mM. When added at this time, only 65% growth inhibition resulted. It appears therefore that confidence can be placed in the ability of INH at a concentration of 0.1 mM to inhibit growth only when it is added to 21-hr cultures.

Summary

When added at a level of 0.1 mM at 21 hr, INH consistently caused essentially complete inhibition of growth; this level and time of addition were therefore used in all subsequent experiments.

Experiment 2

Introduction

The cellulolytic bacteria obtained from the rumen that proliferate under the conditions of the present study utilize ammonia as their source of nitrogen. Given ammonia as the source of nitrogen, the bacteria must first synthesize the amino acids necessary for protein synthesis and concomitant growth. Vitamin B_6, a member of the B-complex synthesized in the rumen (McElroy and Goss, 1939, 1940; Wegner et al., 1940a; and Kon and Porter, 1953), serves as a cofactor for a number of enzymatic reactions involving amino acids. One of these reactions, transamination, is of major importance in the synthesis of amino acids. Although it is probable that transamination reactions are involved in the synthesis of amino acids from ammonia nitrogen by ruminal cellulolytic bacteria, their involvement has never been demonstrated.

Isonicotinic acid hydrazide is known to inhibit transamination and other enzymatic reactions involved in amino acid metabolism that require vitamin B_6 as a cofactor. This compound inhibits the growth of the ruminal cellulolytic bacteria that proliferate in the medium used in the present study. In Experiment 2, runs 21 to 23 were conducted to study the effects of adding three vitamin B_6 forms -- pyridoxal, pyridoxine, or pyridoxamine -- to cultures inhibited with INH.

Experimental

For each run fermentation flasks were prepared and inoculated as previously described. Three flasks were assigned to each treatment group. The appropriate untreated control groups and an INH treated group were included in each run.

Separate aqueous solutions of INH and each of the vitamin B_6 forms

were prepared such that the desired concentration in the fermentation flasks could be attained by addition of 1 ml of the appropriate solution to the appropriate flask. Isonicotinic acid hydrazide was added at 21 hr while the vitamin B_6 forms were added at 0, 21, or 27 hr at levels of 1.0 or 1.5 mM. The fermentations were allowed to proceed until a total of 36 hr had elapsed, at which time all flasks were sampled and their BPN content determined as previously described.

Results

Pyridoxine, pyridoxal, and pyridoxamine, in concentrations 10 or 15 times greater than the concentration of INH employed, were all ineffective in reversing the growth inhibition caused by 0.1 mM INH. This was true regardless of whether they were added 21 hr before (Table 9), at the same time as (Table 10), or 6 hr after (Table 11) the addition of INH. In all cases, the average amount of BPN present in the control flasks at 36 hr was significantly ($P < 0.01$) greater than that present in any of the treatment groups. In all cases except two, the average amount of BPN present at 36 hr in groups to which both a vitamin B_6 form and INH had been added during the fermentation period, was not significantly ($P < 0.01$) different from that present at 36 hr in the group to which INH alone had been added. In the first exception (Table 9), pyridoxal was added to a group of three flasks at 0 hr, i.e. 21 hr prior to the addition of INH. The average amount of BPN present at 36 hr in this group was significantly ($P < 0.01$) less than that present at 36 hr in the group to which INH alone was added at 21 hr. This effect was not observed in groups treated similarly with pyridoxine or pyridoxamine, and suggested that pyridoxal was, in itself, growth inhibitory. In the second exception, the average amount of BPN present at 36 hr was significantly ($P < 0.01$)

greater when pyridoxamine was added 6 hr after INH (Table 11) than that present at 36 hr in the group treated with INH alone. However, the amount of BPN present in this group was still significantly ($P < 0.01$) less than that present in the untreated control at 36 hr. Furthermore, pyridoxamine added before or with INH caused no such difference (Tables 9 and 10).

Table 9

Effect of pyridoxine, pyridoxal, and pyridoxamine on the growth of cultures inhibited by isonicotinic acid hydrazide when the vitamin forms were added 21 hours before the inhibitor

Treatment	BPN present at 36 hr (mg/100 ml)	BPN present at 36 hr as % of control
Control	51.2	100
INH 0.1 mM added at 21 hr	25.7	50
Pyridoxine 1.0 mM added at 0 hr, INH 0.1 mM added at 21 hr	28.1	55
Pyridoxal 1.0 mM added at 0 hr, INH 0.1 mM added at 21 hr	10.4	20
Pyridoxamine 1.0 mM added at 0 hr, INH 0.1 mM added at 21 hr	24.7	48

Table 10

Effect of pyridoxine, pyridoxal, and pyridoxamine on the growth of cultures inhibited with isonicotinic acid hydrazide when the vitamin forms were added with the inhibitor

Treatment	BPN present at 36 hr (mg/100 ml)	BPN present at 36 hr as % of control
Control	52.6	100
INH 0.1 mM added at 21 hr	20.2	38
INH 0.1 mM + pyridoxine, 1.5 mM added at 21 hr	22.0	42
INH 0.1 mM + pyridoxal, 1.5 mM added at 21 hr	21.0	40
INH 0.1 mM + pyridoxamine, 1.5 mM added at 21 hr	20.1	38

Table 11

Effect of pyridoxine, pyridoxal, and pyridoxamine on the growth of cultures inhibited with isonicotinic acid hydrazide when the vitamin forms were added 6 hours after addition of the inhibitor

Treatment	BPN present at 36 hr (mg/100 ml)	BPN present at 36 hr as % of control
Control	52.6	100
INH 0.1 mM added at 21 hr	20.2	38
INH 0.1 mM added at 21 hr, pyridoxine 1.5 mM added at 27 hr	21.7	41
INH 0.1 mM added at 21 hr, pyridoxal 1.5 mM added at 27 hr	22.2	42
INH 0.1 mM added at 21 hr, pyridoxamine 1.5 mM added at 27 hr	24.5	47

To obtain further evidence on the apparent inhibitory effect of pyridoxal (Table 9) and to determine if pyridoxine and pyridoxamine had any effect on growth in the absence of INH, three treatment groups were set up and treated with either pyridoxine, pyridoxal, or pyridoxamine at 0 hr; the fermentations were allowed to continue for 36 hr at which time samples were analyzed for BPN. It was obvious that pyridoxal inhibited growth -- the average amount of BPN present at 36 hr in the pyridoxal-treated group was only 10.0 mg/100 ml (Table 12) as compared to a level of 9.3 mg/100 ml in the 0-hr controls at the time pyridoxal was added. Pyridoxine and pyridoxamine had no effect on growth.

Table 12

Effect of pyridoxine, pyridoxal, and pyridoxamine on the growth of cultures

Treatment	BPN present at 36 hr (mg/100 ml)	BPN present at 36 hr as % of control
Control	51.1	100
Pyridoxine 1.0 mM added at 0 hr	49.7	97
Pyridoxal 1.0 mM added at 0 hr	10.0	20
Pyridoxamine 1.0 mM added at 0 hr	47.3	93

Subsequently, the effect of pyridoxal on growth and on the inhibition of cultures treated with INH was studied using pyridoxal at a concentration 1000 times less than that previously employed. At this concentration (0.001 mM) there was no growth inhibition in the group to which pyridoxal alone was added at 0 hr (Table 13). The average amount of BPN present at 36 hr was not significantly different from that present in the untreated control group at the same time. Results similar to those obtained with 1.0 mM vs. 0.001 mM pyridoxal were reported by Pope (1956) who, working with Mycobacterium tuberculosis H37Rv, found that as the concentration of pyridoxal in the medium increased the growth of this organism was increasingly reduced.

Pyridoxal at a concentration of 0.001 mM did not reverse the growth inhibition resulting from the addition of INH at 21 hr regardless of whether it was added to cultures 21 hr before, at the same time as, or 6 hr after the introduction of INH (Table 13).

Table 13

Effect of 0.001 millimolar pyridoxal in the absence and in the presence of isonicotinic acid hydrazide

Treatment	BPN present at 36 hr (mg/100 ml)	BPN present at 36 hr as % of control
Control	36.9	100
INH 0.1 mM added at 21 hr	17.9	48
Pyridoxal 0.001 mM added at 0 hr	37.4	101
Pyridoxal 0.001 mM added at 0 hr, INH 0.1 mM added at 21 hr	17.7	48
INH 0.1 mM + pyridoxal 0.001 mM added at 21 hr	17.0	46
INH 0.1 mM added at 21 hr, pyridoxal 0.001 mM added at 27 hr	17.8	48

Discussion

From the data presented, no conclusions concerning the mode of action of the INH on the organisms can be drawn. Likewise, since the vitamin B_6 forms did not reverse the action of INH, there is no indication that INH inhibited one or more vitamin B_6 dependent enzyme systems. Possibly the levels at which the vitamin B_6 forms were used were too low to cause any reversal of the inhibition caused by the INH. Pope (1956) has reported that the concentration of pyridoxal required to overcome the growth inhibiting effect of INH on Mycobacterium tuberculosis H37Rv was approximately 300 times greater than the concentration of INH which caused 100% inhibition. However, a review of the literature reveals that when a vitamin B_6 derivative antagonizes the action of INH on an isolated B_6 dependent enzyme, it does so at concentrations equal to or less than the concentrations of INH.

In the initial phases of the present study it was hypothesized that INH might exert its effect by inhibiting transamination reactions and thus inhibit growth by inhibiting the ability of the rumen bacteria to synthesize some or all of the amino acids necessary for protein synthesis. However, in other microorganisms it has been demonstrated that the effect of INH on growth may be separable from its effect on the transaminases of cell-free extracts prepared from the same organisms. Youatt (1958) reported that although INH inhibited the growth of Mycobacterium tuberculosis, it did not affect the transaminase activity of cell-free extracts prepared from cells exposed to INH for 7 days. However, when INH was added to a cell-free extract of this organism, the transaminase activity of the extract was inhibited. Similarly, Hicks (1961) reported that INH inhibited the growth of an Escherichia coli mutant, did not affect the transaminase activity of whole-cell suspensions, and did inhibit the

transaminase activity of cell-free extracts of the organism. Both authors concluded that the growth inhibiting effect of INH was not due to inhibition of transaminase activity but exerted its effect at some point other than an enzymatic reaction requiring vitamin B_6.

It is interesting to note that Boone and Woodward (1953) reported that INH inhibited the growth of Lactobacillus plantarum and Saccharomyces carlsbergensis, and that the inhibition was competitively reversed by pyridoxine, pyridoxal, and pyridoxamine. Both these organisms require an external source of vitamin B_6 for growth. The growth of Mycobacterium tuberculosis and two strains of Escherichia coli, organisms which synthesize their own supply of vitamin B_6, was also inhibited by INH. However, the action of INH on Mycobacterium tuberculosis was not antagonized by the vitamin B_6 forms in concentrations 100 times greater than the effective concentration of INH. In the case of Escherichia coli strains, only partial reversal of the inhibition was achieved and only when large amounts of the vitamin B_6 forms were employed. The bacteria proliferating under the conditions of the present study apparently do not require an external source of vitamin B_6.

Pope (1956) working with Mycobacterium tuberculosis, and Hicks (1961) working with an Escherichia coli mutant have demonstrated that certain amino acids partially antagonize the growth inhibition of whole cells caused by INH. However, this did not appear to be related to any effect of INH on transamination. Hicks (1961) suggested that INH may have prevented the incorporation of certain amino acids into protein. Support for this hypothesis is presented by Tsukamura and Tsukamura (1963). Using isotopically-labeled glutamic acid and glycine, these investigators demonstrated that INH inhibited the incorporation of glutamic acid but not

glycine into the proteins of Mycobacterium jucho and Mycobacterium tuberculosis var. hominis. The conversion of alpha-ketoglutaric acid to glutamic acid was not inhibited by INH.

Bryant et al. (1959) reported that several strains of Bacteroides succinogenes preferentially utilized ammonia as their source of nitrogen in the presence of 18 amino acids, purines, pyrimidines, and all the B-vitamins. Similar observations were reported by Bryant and Robinson (1961) for several strains of Ruminococcus flavefaciens and Ruminococcus albus. These organisms are considered to be responsible for a major portion of the cellulolytic activity in the rumen. For this reason, plus the fact that the bacteria proliferating in the present study are undoubtedly cellulolytic, no attempt was made to determine the effect of amino acids on the inhibitory action of INH. However, attempts were made in Experiment 3 to determine if INH exerted its inhibitory effect by interfering with the organisms' ability to utilize cellulose.

Summary

1) Three vitamin B_6 forms, pyridoxine, pyridoxal, and pyridoxamine, at concentrations of 1.0 or 1.5 mM had no effect on the inhibition caused by INH at a concentration of 0.1 mM when added to cultures 21 hr before, with, or 6 hr after the addition of INH.

2) In the absence of INH, pyridoxal added to cultures at a concentration of 1.0 mM at 0 hr inhibited growth; at a concentration of 0.001 mM pyridoxal did not affect growth.

3) In the absence of INH, pyridoxine or pyridoxamine added to cultures at a concentration of 1.0 mM at 0 hr did not affect growth.

Experiment 3

Introduction

Purified wood cellulose was the sole source of energy, and a major source of carbon in the medium employed in the present study. If INH interferes with the ability of the bacteria to utilize cellulose, growth could be inhibited owing to a deficiency of energy and carbon substrates.

The major end products resulting from the utilization of cellulose by ruminal cellulolytic bacteria are volatile fatty acids (VFA) and CO_2. Experiment 3 was conducted to determine the effect of adding INH to cultures of the ruminal cellulolytic bacteria proliferating under the conditions of the present study on their ability to utilize cellulose as measured by (A) total VFA production and (B) CO_2 production.

A. Total VFA Production

Experimental

The supernatant solutions remaining after the precipitation of bacterial protein were saved in all cases and stored in stoppered test tubes at 4 C. Supernatant solutions from treatment groups to which INH at a level of 0.1 mM had been added at 21 hr in runs 18, 21, and 25 and from the corresponding 21 and 36-hr control groups were selected for total VFA analysis. A 10-ml sample was taken from each of the three supernatant solutions for each treatment or control group. The three samples were pooled and a 25-ml sample of the pooled solution analyzed for total VFA using the method of Neish (1952).

Results

The data for VFA production and for BPN are summarized in Table 14.

Table 14

Estimated production of total volatile fatty acid and bacterial protein nitrogen in runs 18, 21, and 25

Run		18	21	25	Mean
Total VFA			mEq/100 ml		
In 36-hr control		9.16	9.44	8.45	9.02
At 36 hr, INH at 21 hr		5.96	4.20	4.08	4.75
In 21-hr control		3.08	2.48	2.46	2.67
Production in					
Control group 21-36 hr		6.08	6.96	5.99	6.34
Treated group 21-36 hr		2.88	1.72	1.62	2.07
Treated ÷ control x 100	%	47	25	27	33
BPN			mg/100 ml		
In 36-hr control		48.9	52.6	49.2	50.2
At 36 hr, INH at 21 hr		29.0	20.2	19.4	22.9
In 21-hr control		28.0	22.1	20.0	23.4
Production in					
Control group 21-36 hr		20.9	30.5	29.2	26.9
Treated group 21-36 hr		+1.0	-1.9	-0.6	-0.3
Treated ÷ control x 100	%	5	-6	-2	-1

Discussion

It was observed in Experiment 1 that 0.1 mM INH completely inhibited growth as judged by the fact that the amount of BPN present at 36 hr in flasks treated with INH at 21 hr was essentially the same as that present in the 21-hr controls. Similarly, in runs 18, 21, and 25 there was little or no increase in BPN after the addition of INH as compared with the corresponding increases for the control flasks (Table 14). If it is assumed that levels of BPN served as a reasonably accurate indication of the relative numbers of organisms present in treated and untreated flasks, it may also be assumed that VFA production in treated flasks after the intro-

duction of INH arises from the activity of an essentially fixed number of organisms, whereas in the untreated control flasks an increasing population of organisms is involved. Thus, while the mean production of VFA in treated flasks was only 33% of that in the controls (Table 14), it seems reasonable to conclude that to obtain a more accurate indication of the relative VFA producing capacity of the organisms in treated vs. untreated flasks, corrections should be applied for the numbers of organisms in treated vs. control flasks.

Of necessity, any calculation made in an attempt to estimate VFA production per organism in treated vs. control flasks involves assumptions of questionable validity. The calculation adopted is illustrated below using data from run 18.

Treated flasks —

28.0 mg BPN 21 - 36 hr represents N active organisms

VFA production per N organisms, $\frac{2.88}{1}$ = 2.88 mEq

(The refinement of using the value for BPN at 21 hr for treated flasks to represent N organisms is clearly not justifiable.)

Control flasks —

28.0 mg BPN at 21 hr represents N active organisms

48.9 mg BPN at 36 hr represents $\frac{48.9}{28.0}$ x N = 1.75 N organisms

mean number organisms present 21 - 36 hr, $\frac{1\ N + 1.75\ N}{2}$ = 1.375 N

VFA production per N organisms, $\frac{6.08}{1.375}$ = 4.42 mEq

Based on the figures obtained by the above method of calculation, the VFA producing capacity of the organisms in the treated flasks could

be estimated as 65% (2.88/4.42 x 100) of that of those in the control flasks. The corresponding calculated figures for runs 21 and 25 are 42% and 47%. Thus, estimated in this way, the mean VFA production per organism in the treated flasks of runs 18, 21, and 25 was approximately 50% of that in the control flasks vs. a mean of 33% (Table 14, p. 44) for actual production of VFA in treated as compared to control flasks. In any event, if VFA production is accepted as an index of the ability of the bacteria in the cultures to utilize cellulose and BPN production as an index of the numbers of bacteria in the cultures, the results of Part A of Experiment 3 indicate that bacteria present at the time of addition of INH retained an appreciable fraction of their ability to utilize cellulose, but that (in agreement with the results of Experiment 1) they lost their ability to multiply in number.

B. CO_2 Production

Experimental

CO_2 production was measured manometrically, using a standard Warburg apparatus. The single side-arm flasks and the manometers were calibrated with mercury, the respective flask constants calculated, and the final calculations carried out as described by Umbreit, Burris, and Stauffer (1957).

Each Warburg experiment was divided into two parts. Part 1 was designed to study the effect of INH on CO_2 production in Warburg flasks during a 3-hr period beginning 0.5 to 1 hr after the addition of INH to samples of a 21-hr culture, and Part 2 to study CO_2 production by a culture to which INH was added some hours before transfer of samples of this culture to Warburg flasks.

Culture samples for both parts were obtained from the same

Erlenmeyer flask. The samples for Part 1 were taken from the flask at 21 hr. Immediately after these samples were taken, sufficient INH to give a concentration of 0.1 mM in the remaining volume was added to the flask.

Duplicate Warburg flasks for each experimental treatment in Parts 1 and 2 were prepared as outlined below.

Part 1 (INH added 0.5 - 1 hr before 3-hr test period)

Treatment		Culture sample	INH 0.5 mM	Phosphate buffer	$HgCl_2$[1] or NaF[2]	Volume
Control	Flask	4 ml				
	Sidearm			1 ml		5 ml
INH 0.1 mM	Flask	4 ml				
	Sidearm		1 ml			5 ml
Killed control	Flask	4 ml				
	Sidearm				1 ml	5 ml

Part 2 (INH added 6.5 to 7 or 11.5 to 12 hr before 3-hr test period)

INH 0.1 mM	Flask	4 ml				
	Sidearm			1 ml		5 ml
Killed control	Flask	4 ml				
	Sidearm				1 ml	5 ml

[1]Final concentration 0.05 M.

[2]Final concentration 0.10 M.

Each Warburg flask was immediately attached to its manometer, the contents of the flask and sidearm were mixed, and the units set in the water bath at 39 C. The system was then flushed with CO_2 for 30 sec by passing the CO_2 in through the sidearm-stopper gas vent and out the manometer stopcock. The system was then closed and allowed to equilibrate for 30 to 60 min. After the equilibration period, readings were taken at intervals for a period of 3 hr.

The rate of CO_2 production was such that the limits of the manometer were quickly exceeded. To overcome this problem without changing the manometer fluid, the pressure was released and the manometer fluid was adjusted back to the original reference mark (150 mm) after each reading. This was done by restoring the level of the manometer fluid in the flask arm of the manometer to the level which existed before the reading was taken, quickly opening the stopcock to release the pressure, readjusting to the reference mark, and quickly reclosing the stopcock.

Changes in the manometer readings were recorded for each adjustment interval and totaled at the end of each run to obtain the cumulative change.

Results

When added to the culture samples 0.5 to 1 hr before the test, INH significantly ($P<0.01$) reduced the rate at which CO_2 was produced over the ensuing 3-hr period; however, the rate of CO_2 production by samples containing INH was significantly ($P<0.01$) greater than that of those containing either $HgCl_2$ or NaF as bactericidal agents (Table 15). The rate of CO_2 production by samples of cultures to which INH was added several hours before the measurement of CO_2 production was significantly ($P<0.01$) greater than that of samples treated with the bactericides.

Table 15

Effect of 0.1 millimolar isonicotinic acid hydrazide on the production of carbon dioxide by rumen cellulolytic bacteria

	CO_2 production measured over a 3-hr period	
Run	25	26
	µl/min	
Part 1		
Treatment		
Control	3.2	4.0
INH added 0.5-1 hr before test	2.8	3.1
$HgCl_2$, 0.05 M	0.6	
NaF, 0.1 M		0.0
Part 2		
INH added 11.5-12 hr before test	1.27	
INH added 6.5-7 hr before test		2.34
$HgCl_2$, 0.05 M	0.25	
NaF, 0.1 M		0.12

Discussion

The results for CO_2 production by cultures treated with INH are in general agreement with those of Part A in which total VFA production was the criterion of cellulose utilization. If it is assumed, for reasons discussed in Part A, that there was no increase in numbers of organisms in treated flasks after the addition of INH, the decreasing rate of CO_2 production with increasing length of the period of exposure could be regarded as a reflection of progressively decreasing activity of the organisms present when INH was added to the cultures. The results for the effect of the bactericides, $HgCl_2$ and NaF, on CO_2 production vs. those for the effect of INH provide additional evidence that INH was not bactericidal.

Summary

1) Isonicotinic acid hydrazide reduced the total VFA production and rate of CO_2 production of cultures growing in a medium containing cellulose as the source of energy and a source of carbon.

2) The effects of INH on VFA and CO_2 production appear to be attributable more to a lack of cell multiplication in cultures containing INH than to inhibition of cellulose utilization by preformed organisms.

SUMMARY AND DISCUSSION

A medium was devised which gave satisfactory growth of cellulolytic bacteria obtained from the rumen. Constant availability of ammonia nitrogen from $(NH_4)_2SO_4$ and increased availability of the cellulose substrate obtained by heating a suspension of this in an autoclave appeared to be the major factors responsible for the success of this medium.

Isonicotinic acid hydrazide completely inhibited the growth of cultures of rumen cellulolytic bacteria when added to the cultures at a level of 0.1 mM at 21 hr.

Neither pyridoxine, pyridoxal, nor pyridoxamine reversed the inhibition caused by INH when added singly to the cultures either 21 hr before, at the same time as, or 6 hr after the addition of INH.

These findings suggest either that transamination does not occur in the rumen cellulolytic bacteria involved in these experiments or, much more likely in view of the ability of the organisms to use ammonia as their sole nitrogen source, that INH inhibited growth by means other than its known effect on transamination. Other workers have found that although INH inhibited the transaminase activity of cell-free extracts of certain bacteria, the transaminase activity of cell-free extracts prepared from cultures of the same bacteria whose growth had been inhibited with INH was comparable to that of cell-free extracts prepared from untreated cultures.

The rumen cellulolytic bacteria which proliferate in the medium used in this study have the ability to utilize ammonia as their sole source of nitrogen and cellulose as their sole source of energy and major source of carbon, and would be very suitable organisms for use in further

attempts to demonstrate the occurrence of transamination. In view of the findings of other workers, the preparation and study of cell-free extracts of these bacteria might provide more definite means of demonstrating transamination.

Further experiments designed to study the effects of INH on the ability of cultures treated with this compound to utilize cellulose, as measured by the production of total VFA and CO_2, demonstrated that although INH inhibited the growth of such cultures the cells present at the time the INH was added retained an appreciable capacity to utilize cellulose. Cells treated with either $HgCl_2$ or NaF, known bactericides, lost their ability to utilize cellulose as measured by CO_2 production, thus indicating that the effect of INH was not bactericidal.

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